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(54) Title: FIBER OPTIC SENSOR WITH ENCODED MICROSPHERES

(57) Abstract

A microsphere–based analytic chemistry system is disclosed in which microspheres carrying different chemical functionalities may be mixed together while the ability is retained to identify the functionality on each bead using an optically interrogatable encoding scheme. An optical fiber bundle sensor is also disclosed in which the separate microsphere may be optically coupled to discrete fibers or groups of fibers within the bundle. The functionalities are encoded on the separate microspheres using fluorescent dyes and then affixed to wells etched in the end of the bundle. Thus, a single sensor may carry thousands of chemistries. Only those microspheres exhibiting reactions then need to be decoded to identify the corresponding functionality.
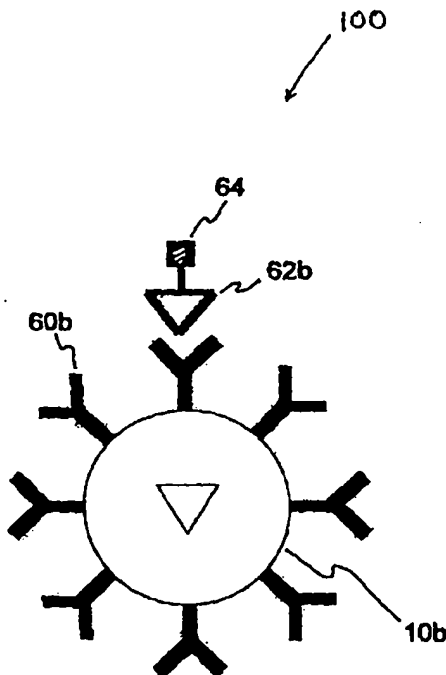
FIBER OPTIC SENSOR WITH ENCODED MICROSPHERES

BACKGROUND OF THE INVENTION

The use of optical fibers and optical fiber strands in
5   combination with light absorbing dyes for chemical analytical
determinations has undergone rapid development, particularly
within the last decade. The use of optical fibers for such
purposes and techniques is described by Milanovich et al.,
"Novel Optical Fiber Techniques For Medical Application",
10   Proceedings of the SPIE 28th Annual International Technical
Symposium On Optics and Electro-Optics, Volume 494, 1980;
Seitz, W.R., "Chemical Sensors Based On Immobilized
Indicators and Fiber Optics" in *C.R.C. Critical Reviews In
Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis,
15   O.S., "Fiber Optical Fluorosensors In Analytical Chemistry"
in *Molecular Luminescence Spectroscopy, Methods and
Applications* (S. G. Schulman, editor), Wiley & Sons, New York
(1988); Angel, S.M., *Spectroscopy* 2(4):38 (1987); Walt, et
al., "Chemical Sensors and Microinstrumentation", *ACS
20   Symposium Series*, Vol. 403, 1989, p. 252, and Wolfbeis, O.S.,
Fiber Optic Chemical Sensors, Ed. CRC Press, Boca Raton, FL,
1991, 2nd Volume.

When using an optical fiber in an *in vitro/in vivo*
sensor, one or more light absorbing dyes are located
25   near its distal end. Typically, light from an
appropriate source is used to illuminate the dyes
through the fiber's proximal end. The light propagates
along the length of the optical fiber; and a portion of
this propagated light exits the distal end and is
30   absorbed by the dyes. The light absorbing dye may or
may not be immobilized; may or may not be directly
attached to the optical fiber itself; may or may not be
suspended in a fluid sample containing one or more

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analytes of interest; and may or may not be retainable
for subsequent use in a second optical determination.

Once the light has been absorbed by the dye, some
light of varying wavelength and intensity returns,
5   conveyed through either the same fiber or collection
fiber(s) to a detection system where it is observed and
measured.   The interactions between the light conveyed
by the optical fiber and the properties of the light
absorbing dye provide an optical basis for both
10   qualitative and quantitative determinations.

Of the many different classes of light absorbing
dyes which conventionally are employed with bundles of
fiber strands and optical fibers for different
analytical purposes are those more common compositions
15   that emit light after absorption termed "fluorophores"
and those which absorb light and internally convert the
absorbed light to heat, rather than emit it as light,
termed "chromophores."

Fluorescence is a physical phenomenon based upon the
20   ability of some molecules to absorb light (photons) at
specified wavelengths and then emit light of a longer
wavelength and at a lower energy.   Substances able to
fluoresce share a number of common characteristics: the
ability to absorb light energy at one wavelength λ_{ab}; reach
25   an excited energy state; and subsequently emit light at
another light wavelength, λ_{em}.   The absorption and
fluorescence emission spectra are individual for each
fluorophore and are often graphically represented as two
separate curves that are slightly overlapping.   The same
30   fluorescence emission spectrum is generally observed
irrespective of the wavelength of the exciting light and,
accordingly, the wavelength and energy of the exciting light
may be varied within limits; but the light emitted by the

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fluorophore will always provide the same emission spectrum.
Finally, the strength of the fluorescence signal may be
measured as the quantum yield of light emitted. The
fluorescence quantum yield is the ratio of the number of
5 photons emitted in comparison to the number of photons
initially absorbed by the fluorophore. For more detailed
information regarding each of these characteristics, the
following references are recommended: Lakowicz, J. R.,
Principles of Fluorescence Spectroscopy, Plenum Press, New
10 York, 1983; Freifelder, D., Physical Biochemistry, second
edition, W. H. Freeman and Company, New York, 1982;
"Molecular Luminescence Spectroscopy Methods and
Applications: Part I" (S.G. Schulman, editor) in *Chemical
Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of*
15 *Luminescence*, Stepanov and Gribkovskii, Iliffe Books, Ltd.,
London, 1968.

In comparison, substances which absorb light and do not
fluoresce usually convert the light into heat or kinetic
energy. The ability to internally convert the absorbed light
20 identifies the dye as a "chromophore." Dyes which absorb
light energy as chromophores do so at individual wavelengths
of energy and are characterized by a distinctive molar
absorption coefficient at that wavelength. Chemical analysis
employing fiber optic strands and absorption spectroscopy
25 using visible and ultraviolet light wavelengths in
combination with the absorption coefficient allow for the
determination of concentration for specific analytes of
interest by spectral measurement. The most common use of
absorbance-measurement via optical fibers is to determine
30 concentration which is calculated in accordance with Beers'
law; accordingly, at a single absorbance wavelength, the
greater the quantity of the composition which absorbs light
energy at a given wavelength, the greater the optical density

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for the sample. In this way, the total quantity of light
absorbed directly correlates with the quantity of the
composition in the sample.

5   Many of the recent improvements employing optical fiber
sensors in both qualitative and quantitative analytical
determinations concern the desirability of depositing and/or
immobilizing various light absorbing dyes at the distal end
of the optical fiber. In this manner, a variety of different
optical fiber chemical sensors and methods have been reported
10   for specific analytical determinations and applications such
as pH measurement, oxygen detection, and carbon dioxide
analyses. These developments are exemplified by the
following publications: Freeman, et al., *Anal Chem.* 53:98
(1983); Lippitsch et al., *Anal. Chem. Acta.* 205:1, (1988);
15   Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al.,
Anal. Chem. 59:437 (1987); Lubbers et al., *Sens. Actuators*
1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et
al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.*
56:16A-34A (1984); Peterson, et al., *Anal. Chem.* 52:864
20   (1980): Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et
al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem.*
Acta. 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199
(1984); Wolfbeis, O.S., "Fiber Optic Chemical Sensors", *Ed.*
CRC Press, Boca Raton, FL, 1991, 2nd Volume; and Pantano, P.,
25   Walt, D.R., *Anal. Chem.,* 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed
that permit the use of multiple dyes with a single, discrete
fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264
to Walt, *et al.* disclose systems for affixing multiple,
30   different dyes on the distal end of the bundle, the teachings
of each of these patents being incorporated herein by this
reference. The disclosed configurations enable separate
optical fibers of the bundle to optically access individual

dyes. This avoids the problem of deconvolving the separate
signals in the returning light from each dye, which arises
when the signals from two or more dyes are combined, each dye
being sensitive to a different analyte, and there is
5 significant overlap in the dyes' emission spectra.

SUMMARY OF THE INVENTION

The innovation of the two previous patents was the
placement of multiple chemical functionalities at the end of
a single optical fiber bundle sensor. This configuration
10 yielded an analytic chemistry sensor that could be remotely
monitored via the typically small bundle. The drawback,
however, was the difficulty in applying the various
chemistries associated with the chemical functionalities at
the sensor's end; the functionalities were built on the
15 sensor's end in a serial fashion. This was a slow process,
and in practice, only tens of functionalities could be
applied.

The present design is based on two synergistic
inventions: 1) the development of a bead-based analytic
20 chemistry system in which beads, also termed microspheres,
carrying different chemical functionalities may be mixed
together while the ability is retained to identify the
functionality of each bead using an optically interrogatable
encoding scheme; and 2) an optical fiber bundle sensor in
25 which the separate beads or microspheres may be optically
coupled to discrete fibers or groups of fibers within the
bundle. Each invention has separate applications but, when
implemented together, yields an optical fiber sensor that can
support large numbers, thousands or more, of separate
30 chemical functionalities, which is relatively easy to
manufacture and use.

In general, according to one aspect, the invention
concerns an analytic chemistry system that comprises a

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population of beads or microspheres. Within the population
are separate subpopulations, each of which carries chemical
functionality which changes an optical signature of the beads
in a presence of targeted analytes. This signature change
5   can occur via many different mechanisms. A few examples
include the binding of a dye-tagged analyte to the bead, the
production of a dye species on or near the beads, the
destruction of an existing dye species, a change in optical
signal upon analyte interaction with dye on bead, or any
10 other optical interrogatable event. Although the
subpopulations may be randomly mixed together, the chemical
functionality on each bead is determined via an optical
signature which is encoded with a description of the chemical
functionality. As a result, by observing whether the optical
15 signature of a particular bead is exhibiting a change, or
not, and then decoding the signature for the functionality of
the bead, the presence or not of the analyte targeted by the
functionality may be determined.

        In specific embodiments, the beads are encoded using
20 dyes that are preferably entrapped within the beads, the
chemical functionality being added on surfaces. The dyes may
be chromophores or phosphors but are preferably fluorescent
dyes, which due to their strong signals provide a good
signal-to-noise ratio for decoding. The encoding can be
25 accomplished in a ratio of at least two dyes, although more
encoding dimensions may be added in the size of the beads,
for example.

        According to another aspect, the invention also concerns
an analytic chemistry fiber optic bundle sensor. This sensor
30 has a population of beads carrying chemical functionalities
at, on or near, a distal end of the bundle. The ability to
monitor optical signature changes associated with individual
or multiple beads is provided by coupling those signature

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changes into separate optical fibers or groups of fibers of
the bundle for transmission to the proximal end where
analysis is performed either manually, by the user, or
automatically, using image processing techniques.

5   In the preferred embodiment, each one of the beads is
located within separate wells formed at terminal ends of
optical fibers of the bundle. These microwells are formed by
anisotropic etching of the cores of the optical fibers with
respect to the cladding.

10   Also in the preferred embodiment, the population of
beads includes separate subpopulations, typically randomly
distributed in an array across the bundle end, each
subpopulation carrying a different chemical functionality and
an optically interrogatable code descriptive of the chemical
15 functionality.

       Although each sensor is different insofar that it has a
different distribution of the subpopulations of beads within
its wells, only those beads that exhibit a positive optical
response or signature change need to be decoded. Therefore,
20 the burden is placed on the analysis rather than on sensor
manufacture. Moreover, since the beads and fibers in the
array can be monodisperse, the fluorescent regions arising
from signal generation are extremely uniform and can be
analyzed automatically using commercially available
25 microscopy analysis software, such image processing software
is capable of defining different spectral regions
automatically and counting the number of segments within each
region in several seconds.

       The above and other features of the invention including
30 various novel details of construction and combinations of
parts, and other advantages, will now be more particularly
described with reference to the accompanying drawings and
pointed out in the claims. It will be understood that the

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particular method and device embodying the invention are shown by way of illustration and not as a limitation of the invention. The principles and features of this invention may be employed in various and numerous embodiments without

5 departing from the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings, reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale; emphasis has instead

10 been placed upon illustrating the principles of the invention. Of the drawings:

Fig. 1 is a schematic diagram illustrating the optical signature encoding and chemical functionalizing of the microspheres according to the present invention;

15 Fig. 2 is a process diagram describing the preparation, encoding, and functionalizing of the microspheres of the present invention;

Fig. 3 is a schematic diagram illustrating a microsphere system including microspheres with different chemical

20 functionalities and encoded descriptions of the functionalities;

Fig. 4 is a schematic diagram of the inventive fiber optic sensor and associated instrumentation and control . system;

25 Fig. 5A and 5B are micrographs illustrating the preferred technique for attaching or affixing the microspheres to the distal end of the optical fiber bundle;

Fig. 6 is a process diagram describing well formation in the optical fiber bundle and affixation of the microspheres

30 in the wells;

Figs. 7A and 7B are micrographs showing the array of microspheres in their corresponding wells prior and subsequent to physical agitation, tapping and air pulsing,

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demonstrating the electrostatic binding of the microspheres
in the wells;

Figs. 8A, 8B, and 8C are micrographs from alkaline
phosphatase microspheres when exposed to fluorescein di-
5   phosphate, at the fluorescein emission wavelength, at an
encoding wavelength for DiIC, and at an encoding wavelength
for TRC, respectively;

Figs. 9A and 9B are micrographs showing the optical
signal from β-galactosidase microspheres when exposed to
10 fluorescein β-galactopyranoside at the fluorescein emission
wavelength and at an encoding wavelength for DiIC,
respectively; and

Fig. 10A and 10B are micrographs showing the optical
response from xrabbit antibody microspheres prior to and
15 post, respectively, exposure to fluorescein labeled antigens.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

1.   Microspheres:

Fig. 1 illustrates the construction of a bead or
microsphere 10 according to the principles of the present
20 invention.   In common with the prior art, the microsphere 10
is given a chemical functionality 12, which is typically
applied to the microsphere's surface.   The chemical
functionality is designed so that in the presence of the
analyte(s) to which it is targeted, an optical signature of
25 the microsphere, possibly including region surrounding it, is
changed.

The bead or microsphere need not be spherical, irregular
beads may be used.   They are typically constructed from
plastic or ceramic, and bead sizes ranging from nanometers,
30 e.g., 500 nm, to millimeters, e.g., 1 mm, may be used.
Moreover, various bead chemistries may be used.   Beads or
microspheres comprising methylstyrene, polystyrene, acrylic
polymer, latex, paramagnetic, thoria sol, carbon, graphite,

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and titanium dioxide are various possibilities. *"Microsphere Detection Guide"* from Bangs Laboratories, Fishers IN. is a helpful guide.

The inventive microsphere has an optical signature that

5   is encoded with a description of the chemical functionality.

In the preferred embodiment, reporter dyes 14 are added to the microsphere 10 with the encoding occurring in the ratio of two or more dyes. The reporter dyes 14 may be chromophore-type. Fluorescent dyes, however, are preferred

10 because the strength of the fluorescent signal provides a better signal-to-noise ratio when decoding. Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate

15 the reporter dye's signature and detector sensitivity.

Fig. 2 is a process diagram illustrating the preparation of the microspheres. In step 50, an aliquot of stock microspheres are vacuum filtered to produce a dry cake. In one implementation, microspheres of methylstyrene (87%) and

20 divinylbenzene (13%) are used that have a 3.1 micrometer (µm) diameter.

The microspheres may be purchased with the desired chemical functionalities already present. A large selection of such pre-prepared microspheres are currently available

25 from a number of commercial vendors. Alternatively, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres are listed in Table I.

30   The dry cake is then broken apart and a dye solution added to it in step 52 to encode optical signatures of the microspheres with information concerning the intended surface chemical functionalities. Dyes may be covalently bonded to

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the microspheres' surface, but this consumes surface binding
sites desirably reserved for the chemical functionalities.
Preferably, the microspheres are placed in a dye solution
comprising a ratio of two or more fluorescent reporter dyes
5   dissolved in an organic solvent that will swell the
microspheres, e.g., dimethylformamide (DMF). The length of
time the microspheres are soaked in the dye solution will
determine their intensity and the broadness of the ratio
range. Longer times yield higher intensities, but broader
10 ratio ranges.

Table I

SURFACE CHEMISTRY	NAME
NH_2	Amine
COOH	Carboxylic Acid
CHO	Aldehyde
CH_2-NH_2	Aliphatic Amine
CO NH_2	Amide
CH_2-Cl	Chloromethyl
$CONH-NH_2$	Hydrazide
OH	Hydroxyl
SO_4	Sulfate
SO_3	Sulfonate
Ar NH_2	Aromatic Amine

In an exemplary two dye system, Texas Red Cadaverine
(TRC) is used, which is excited at λ_{ab}=580 mm and emits at
15 λ_{em}=630 mm, in combination with indodicarbocyanine (DiIC):

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610/670 ($\lambda_{ab}/\lambda_{em}$). Generally, dyes are selected to be
compatible with the chemistries involved in the analysis and
to be spectrally compatible. The emission wavelengths of the
dyes should not overlap the regions of the optical spectrum
5   in which the chemical functionalities induce changes in the
microsphere signatures. This avoids deconvolution problems
associated with determining signal contributions based on the
presence of both the analyte and the encoding dye ratios
contributing to an overlapping emission spectral region.

10   Examples of other dyes that can be used are Oxazin
(662/705), IR-144 (745/825), IR-140 (776/882), IR-125
(786/800) from Exiton, and Bodipy 665/676 from Molecular
Probes, and Naphthofluorescein (605/675) also from Molecular
Probes. Lanthide may also be used. Fluorescent dyes
15 emitting in other than the near infrared may also be used.
Chromophore dyes are still another alternative that produce
an optically interrogatable signature, as are more exotic
formulations using Raman scattering-based dyes or polarizing
dyes, for example.

20   The ability of a particular dye pair to encode for
different chemical functionalities depends on the resolution
of the ratiometric measurement. Conservatively, any dye pair
should provide the ability to discriminate at least twenty
different ratios. The number of unique combinations of two
25 dyes made with a particular dye set is shown in the following
Table II.

Table II

Number of dyes in set	Combinations possible
3	3
4	6
5	10
6	15

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Thus, using six dyes and twenty distinct ratios for each
dye pair, 300 separate chemical functionalities may be
encoded in a given population of microspheres. Combining
more than two dyes provides additional diversity in the
5 encoding combinations.

In step 54, the microspheres are vacuum filtered to
remove excess dye. The microspheres are then washed in water
or other liquid that does not swell the microspheres, but in
which the dyes are still soluble. This allows the residual
10 dye to be rinsed off without rinsing the dye out of the
microspheres.

In step 56, the chemical functionality is attached to
the microsphere surface chemistries if not already present.
It should be understood that surface chemistries may be
15 present throughout the microsphere's volume, and not limited
to the physical circumferential surface.

In the prior art, a large spectrum of chemical
functionalities have been manifest on microspheres that
produce optically interrogatable changes in the presence of
20 the targeted analyte. These functionalities include four
broad classifications of microsphere sensors: 1) basic
indicator chemistry sensors; 2) enzyme-based sensors; 3)
immuno-based sensors; and 3) geno-sensors.

A large number of basic indicator microsphere sensors
25 have been previously demonstrated. Examples include:

Table III

TARGET ANALYTE	CHEMICAL FUNCTIONALITY	NOTES ($\lambda_{ex}/\lambda_{em}$)
pH Sensors based on:	seminaphthofluoresceins	e.g., carboxy-SNAFL
	seminaphthorhodafluors	e.g., carboxy-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	
	fluorescein	

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CO2 Sensors based On:	seminaphthofluoresceins	e.g., carboxy-SNAFL
	seminaphthorhodafluors	e.g., carbody-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	
Metal Ions Sensors based on:	desferriozamine B	e.g., Fe
	cyclen derivatives	e.g., Cu, Zn
	derivatized peptides	e.g., FITC-Gly-Gly-His, and FITC-Gly His, Cu,Zn
	fluorexon (calcine)	e.g., Ca, Mg, Cu,Pb, Ba
	calcine blue	e.g., Ca, Mg, Cu
	methyl calcine blue	e.g., Ca, Mg, Cu
	ortho-dianisidine tetracetic acid (ODTA)	e.g., Zn
	bis-salicylidene ethylenediamine (SED)	e.g., Al
	N-(6-methoxy-8-quinolyl-p-toluenesulfonamine (TSQ)	e.g., Zn
	Indo-1	e.g., Mn, Ni
	Fura-2	e.g., Mn, Ni
	Magnesium Green	e.g., Mg, Cd, Tb
O_2	Siphenylisobenzofuran	409/476
	Methoxyvinyl pyrene	352/401
Nitrite	diaminonaphthaline	340/377
NO	luminol	355/411
	dihydrohodamine	289/none
Ca^{2+}	Bis-fura	340/380
	Calcium Green	visible light/530
	Fura-2	340/380
	Indo-1	405/485
	Fluo-3	visible light/525
	Rhod-2	visible light/570
Mg^{2+}	Mag-Fura-2	340/380
	Mag-Fura-5	340/380
	Mag-Indo-1	405/485

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	Magnesium Green	475/530
	Magnesium Orange	visible light/545
Zn²⁺	Newport Green	506/535
TSQ	Methoxy-Quinobyl	334/385
Cu⁺	Phen Green	492/517
Na⁺	SBFI	339/565
	SBFO	354/575
	Sodium Green	506/535
K⁺	PBPI	336/557
Cl⁻	SPQ	344/443
	MQAE	350/460

Each of the chemicals listed in Table III directly produces an optically interrogatable signal or optical signature change in the presence of the targeted analyte.

5   Enzyme-based microsphere sensors have also been demonstrated and could be manifest on microspheres. As shown in Table IV, examples include:

Table IV

SENSOR TARGET	CHEMICAL FUNCTIONALITY
Glucose Sensor	glucose oxidase (enz.) + O₂-sensitive dye (see Table I)
Penicillin Sensor	penicillinase (enz.) + pH-sensitive dye (see Table I)
Urea Sensor	urease (enz.) + pH-sensitive dye (see Table I)
Acetylcholine Sensor	acetylcholinesterase (enz.) + pH-sensitive dye (see Table I)

10

Generally, the induced change in the optical signature due to the presence of the enzyme-sensitive chemical analyte

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occurs indirectly in this class of chemical functionalities.
The microsphere-bound enzyme, e.g., glucose oxidase,
decomposes the target analyte, e.g., glucose, consume a co-
substrate, e.g., oxygen, or produce some by-product, e.g.,
5 hydrogen peroxide. An oxygen sensitive dye is then used to
trigger the signature change.

    Techniques for immobilizing enzymes on microspheres, are
known in the prior art. In one case, NH_2 surface chemistry
microspheres are used. Surface activation is achieved with a
10 2.5% glutaraldehyde in phosphate buffered saline (10 mM)
providing a pH of 6.9. (138 mM NaCl, 2.7 mM, KCl). This is
stirred on a stir bed for approximately 2 hours at room
temperature. The microspheres are then rinsed with ultrapure
water plus 0.01% tween 20 (surfactant) -0.02%, and rinsed
15 again with a pH 7.7 PBS plus 0.01% tween 20. Finally, the
enzyme is added to the solution, preferably after being
prefiltered using a 0.45µm amicon micropure filter.

    Immuno-based microsphere sensors have been demonstrated
for the detection for environmental pollutants such as
20 pesticides, herbicides, PCB's and PAH's. Additionally, these
sensors have also been used for diagnostics, such as
bacterial (e.g., leprosy, cholera, lyme disease, and
turburculosis), viral (e.g., HIV, herpes simplex,
cytomegalovirus), fungal (e.g., aspergillosis, candidiasis,
25 cryptococcoses), Mycoplasmal (e.g., mycoplasmal pneumonia),
Protozoal (e.g., amoebiasis, toxoplasmosis), Rickettsial
(e.g., Rock Mountain spotted fever), and pregnancy tests.

    Microsphere genosensors have also been demonstrated.
These are typically constructed by attaching a probe sequence
30 to the microsphere surface chemistry, typically via an NH_2
group. A fluorescent dye molecule, e.g., fluorescein, is
attached to the target sequence, which is in solution. The
optically interrogatable signature change occurs with the

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binding of the target sequences to the microsphere. This
produces a higher concentration of dye surrounding the
microsphere than in the solution generally. A few
demonstrated probe and target sequences, see Ferguson, J.A.
5   et al. Nature Biotechnology, Vol. 14, Dec. 19996, are listed
below in Table V.

        Alternatively, upon binding of the target sequences, an
intercalating dye (e.g., ethidium bromide) can be added
subsequently to signal the presence of the bound target to
10   the probe sequence.

Table V

PROBE SEQUENCES	TARGET SEQUENCES
B-glo(+) (segment of human B-globin)5'-NH$_2$-(CH$_2$)$_6$-)TT TTT TTT TCA ACT TCA TCC ACG TTC ACC-3	B-glo(+)-CF 5'-Fluorescein-TC AAC GTG GAT GAA GTT C-3'
IFNG(interferon gamma 1)5'-NH$_2$-(CH$_2$)$_6$-T$_{12}$-TGG CTT CTC TTG GCT GTT ACT-3'	IFNG-CF 5'-Fluorescein-AG TAA CAG CCA AGA GAA CCC AAA-3'
IL2(interleukin-2)5'-NH$_2$-(CH$_2$)$_6$-T$_{12}$- TA ACC GAA TCC CAA ACT CAC CAG-3'	IL2-CF 5'-Fluorescein-CT GGT GAG TTT GGG ATT CTT GTA-3'
IL4 (interleukin-4)5'NH$_2$-(CH$_2$)$_6$-T$_{12}$-CC AAC TGC TTC CCC CTC TGT-3'	IL4-CF 5 '-Fluorescein-AC AGA GGG GGA AGC AGT TGG-3'
IL6(interleukin-6)5'NH$_2$-(CH$_2$)$_6$-T$_{12}$-GT TGG GTC AGG GGT GGT TAT T-3'	IL6-CF 5'-Fluorescein - AA TAA CCA CCC CTG ACC CAA C-3'

        Fig. 3 illustrates the construction of an analytic
chemistry system 100 from microspheres that have different
15   chemical functionalities. Subpopulations of microspheres are
represented as 10a,10b,10c carrying respective probe
sequences 60a,60b,60c, as exemplary functionalities. These
subpopulations, once manufactured, are combined and mixed
with each other. The resulting combined population contains

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microspheres with randomly distributed individual chemical
functionalities.

Typically, mixing microspheres with different
functionalities results in the loss of information regarding
5   the selectivity for each of the corresponding target
sequences. In a solution of microspheres with each of the
probe sequences 60a, 60b, and 60c, it is possible to
determine that at least one of the target sequences 62a, 62b,
and 62c is present when a fluorescent marker dye 64
10   concentration is observed on the microspheres 10. There is
no way, however, to determine which probe sequence 60a, 60b,
and 60c is generating the activity since the information
concerning which microsphere contained which probe sequence
was lost when the subpopulations were mixed.

15   In contrast, in the present invention, each microsphere
in each subpopulation is encoded with a common optical
signature. In the illustrated example, the subpopulation
represented by microsphere 10a has a two reporter dye ratio
of 10:1; the subpopulation of microspheres 10b has a ratio of
20   1:1 of the same reporter dyes, and subpopulation of
microspheres 10c has a ratio of 1:10 of the reporter dyes.

Thus, the randomly mixed subpopulations of microspheres
are useful as an analytic chemistry system based on each of
the carried chemical functionalities 60a-60c separately. The
25   system 100 is added to an analyte of interest to which some
of the chemical functionalities may interact. Any
interaction changes the optical response of the corresponding
microspheres by, for example, binding a fluorescent dye 64 to
the microspheres. By identifying the chemical
30   functionalities of the microsphere in which the optical
signature has changed, using the encoded dye combinations,
information regarding the chemical identity and concentration
of the analyte may be gained based upon the interaction or

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noninteraction of each functionality contained in the probe
100.

The microspheres exhibiting activity or changes in their
optical signature may be identified by utilizing a somewhat
5   "manual" approach of observing the individual microspheres
through a microscope. Decoding can also be performed
manually, depending on the particular reporter dyes used. It
may be helpful to use optical aids such as light filters to
observe the light from the microspheres at emission
10   wavelengths of the reporter dyes. While this approach is
possible, in the preferred embodiment, the analytic chemistry
microsphere system is used with the inventive optical fiber
sensor.

2. Optical Fiber Sensor:
15   Fig. 4 is a schematic block diagram showing the
inventive optical fiber sensor 200 and associated control
system 210. The fiber optic sensor 200 comprises a fiber
optic bundle 202, that is constructed from separately clad
fibers so that light does not mix between fibers. The
20   microsphere system 100 is attached to the bundle's distal end
212, with the proximal end 214 being received by a z-
translation stage 216 and x-y micropositioner 218. These two
components act in concert to properly position the proximal
end 214 of the bundle 202 for a microscope objective lens
25   220. Light collected by the objective lens 220 is passed to
a reflected light fluorescence attachment with three pointer
cube slider 222. The attachment 222 allows insertion of
light from a 75 Watt Xe lamp 224 through the objective lens
220 to be coupled into the fiber bundle 202. The light from
30   the source 224 is condensed by condensing lens 226, then
filtered and/or shuttered by filter and shutter wheel 228,
and subsequently passes through a ND filter slide 230.

20

Light returning from the distal end 212 of the bundle
202 is passed by the attachment 222 to a magnification
changer 232 which enables adjustment of the image size of the
fiber's proximal or distal end. Light passing through the
5   magnification changer 232 is then shuttered and filtered by a
second wheel 234. The light is then imaged on a charge
coupled device (CCD) camera 236. A computer 238 executes
imaging processing software to process the information from
the CCD camera 236 and also possibly control the first and
10   second shutter and filter wheels 228, 234. The
instrumentation exclusive of the fiber sensor 200, i.e., to
the left of the proximal end of the bundle 202 is discussed
more completely by Bronk, et al., Anal. Chem. 1995, Vol. 67,
number 17, pp. 2750-2752.
15   The microsphere system 100 may be attached to the distal
end of the optical fiber bundle using a variety of compatible
processes. It is important that the microspheres are located
close to the end of the bundle. This ensures that the light
returning in each optical fiber predominantly comes from only
20   a single microsphere. This feature is necessary to enable
the interrogation of the optical signature of individual
microspheres to identify reactions involving the
microsphere's functionality and also to decode the dye ratios
contained in those microspheres. The adhesion or affixing
25   technique, however, must not chemically insulate the
microspheres from the analyte.
Figs. 5A and 5B are micrographs of the distal end 212 of
the bundle 202 illustrating the preferred technique for
attaching the microspheres 10 to the bundle 202. Wells 250
30   are formed at the center of each optical fiber 252 of the
bundle 202. As shown in Fig. 5B, the size of the wells 250
are coordinated with the size of the microspheres 10 so that
the microspheres 10 can be placed within the wells 250.

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Thus, each optical fiber 252 of the bundle 202 conveys light
from the single microsphere 10 contained in its well.
Consequently, by imaging the end of the bundle 202 onto the
CCD array 236, the optical signatures of the microspheres 10
5   are individually interrogatable.

Fig. 6 illustrates how the microwells 250 are formed and
microspheres 10 placed in the wells. A 1 mm hexagonally-
packed imaging fiber contains approximately 20,600 individual
optical fibers that have cores approximately 3.7μm across
10 (Part No. ET26 from Galileo Fibers). Typically, the cores of
each fiber are hexagonally shaped as a result of glass
hardness and drawing during fiber fabrication. In some
cases, the shape can be circular, however.

In step 270, both the proximal and distal ends 212,214
15 of the fiber bundle 202 are successively polished on 12 μm,
9 μm, 3 μm, 1 μm, and 0.3 μm lapping films. Subsequently,
the ends can be inspected for scratches on an atomic force
microscope. In step 272, etching is performed on the distal
end 212 of the bundle 202. A solution of 0.2 grams NH_4F
20 (ammonium fluoride) with 600 μl dH_2O and 100 μl of HF
(hydrofluoric acid), 50% stock solution, may be used. The
distal end 212 is etched in this solution for a specified
time, preferably approximately 80 seconds.

Upon removal from this solution, the bundle end is
25 immediately placed in deionized water to stop any further
etching in step 274. The fiber is then rinsed in running tap
water. At this stage, sonication is preferably performed for
several minutes to remove any salt products from the
reaction. The fiber is then allowed to air dry.
30 The foregoing procedure produces wells by the
anisotropic etching of the fiber cores 254 favorably with
respect to the cladding 256 for each fiber of the bundle 202.

22

The wells have approximately the diameter of the cores 254,
3.7 µm. This diameter is selected to be slightly larger than
the diameters of the microspheres used, 3.1µm, in the
example. The preferential etching occurs because the pure
5   silica of the cores 254 etches faster in the presence of
hydrofluoric acid than the germanium-doped silica claddings
256.

        The microspheres are then placed in the wells 250 in
step 276 according to a number of different techniques. The
10 placement of the microspheres may be accomplished by dripping
a solution containing the desired randomly mixed
subpopulations of the microspheres over the distal end 212,
sonicating the bundle to settle the microspheres in the
wells, and allowing the microsphere solvent to evaporate.
15 Alternatively, the subpopulations could be added serially to
the bundle end. Microspheres 10 may then be fixed into the
wells 250 by using a dilute solution of sulfonated Nafion
that is dripped over the end. Upon solvent evaporation, a
thin film of Nafion was formed over the microspheres which
20 holds them in place. This approach is compatible for fixing
microspheres for pH indication that carry FITC functionality.
        The resulting array of fixed microspheres retains its pH
sensitivity due to the permeability of the sulfonated Nafion
to hydrogen ions. This approach, however, can not be
25 employed generically as Nafion is impermeable to most water
soluble species. A similar approach can be employed with
different polymers. For example, solutions of polyethylene
glycol, polyacrylamide, or polyhydroxymethyl methacrylate
(polyHEMA) can be used in place of Nafion, providing the
30 requisite permeability to aqueous species.

        An alternative fixation approach employs microsphere
swelling to entrap each microsphere 10 in its corresponding
microwell 250. In this approach, the microspheres are first

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distributed into the microwells 250 by sonicating the microspheres suspended in a non-swelling solvent in the presence of the microwell array on the distal end 212. After placement into the microwells, the microspheres are

5   subsequently exposed to an aqueous buffer in which they swell, thereby physically entrapping them, analogous to muffins rising in a muffin tin.

One of the most common microsphere formations is tentagel, a styrene-polyethylene glycol co-polymer. These

10 microspheres are unswollen in nonpolar solvents such as hexane and swell approximately 20-40% in volume upon exposure to a more polar or aqueous media. This approach is extremely desirable since it does not significantly compromise the diffusional or permeability properties of the microspheres

15 themselves.

Figs. 7A and 7B show polymer coated microspheres 12 in wells 250 after their initial placement and then after tapping and exposure to air pulses. Figs. 7A and 7B illustrate that there is no appreciable loss of microspheres

20 from the wells due to mechanical agitation even without a specific fixing technique. This effect is probably due to electrostatic forces between the microspheres and the optical fibers. These forces tend to bind the microspheres within the wells. Thus, in most environments, it may be unnecessary

25 to use any chemical or mechanical fixation for the microspheres.

In alternative embodiments, additional encoding parameters can be added, such as microsphere size. If a number of sensors needed exceeds a few hundred, it is

30 possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters. With different diameters, the largest wells

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can be filled with the largest microspheres and then moving
onto progressively smaller microspheres in the smaller wells
until all size wells are then filled. In this manner, the
same dye ratio could be used to encode microspheres of
5   different sizes thereby expanding the number of different
oligonucleotide sequences or chemical functionalities present
in the array.

EXPERIMENTAL RESULTS

1.   Enzyme-Based Sensor:

10   Subpopulation A

          Chemical functionality: Alkaline phosphatase

          Target substrate: fluorescein diphosphate (FDP)

          Reported dye ratio: 1:1 ratio of DiIC:TRC, where DiIC

is 1,1',3,3,3',3'-hexamethyl-indodicarbocyanine iodide and

15 TRC is Texas Red cadaverine

          A range of ratios of light intensities are selected that
are representative of the optical signature for the dye ratio
of the subpopulation based on the quantum yield of the two
dyes.   The optical signature for this subpopulation is:

20
$$\frac{DiIC\ \lambda\ intensity - ave.DiIC\ background}{TRC\ \lambda\ intensity - ave.TRC\ background} = 0.847 \pm 0.23.$$

          Subpopulation B

          Chemical functionality: B-Galactosidase;

          Target substrate: fluorescein di-B-galactopyranoside
          (FDG)

25   Reporter dye ratio: 10:1 ratio of DiIC:TRC which
          translates to an optical signature of:

$$\frac{DiIC\ \lambda\ intensity - ave.DiIC\ background}{TRC\ \lambda\ intensity - ave.TRC\ background} = 4.456 \pm 1.27..$$

          Subpopulation C

          Chemical functionality: B-glucuronidase

30   Target substrate: fluorescein di-B-D-glucuronide
          (FDGicu).

26

Reporter dye ratio: 1:10 ratio of DiIC:TRC, which translates to an optical signature of:

$$\frac{DiIC\ \lambda\ intensity - ave.\ DiIC\ background}{TRC\ \lambda\ intensity - ave.\ TRC\ background} = 0.2136 \pm 0.03\ ..$$

When the microsphere populations are in the presence of
5   one or more of the substrates, the respective enzymes on the microspheres catalyze the breakdown of the substrates producing fluorescein which is fluorescent, emitting light at 530 nanometers when excited at 490 nm. The production of fluorescein localized to particular beads is then monitored.
10   In this approach, the localization of fluorescein around the microspheres is increased by using a substrate solution of 90% glycerol and 10% substrate. The glycerol prevents the generated fluorescein from diffusing away from the microsphere reaction sites.
15   During the experiment, images in the encoded wavelengths are first taken. Since both DiIC and TRC are excited at 577 nm. Each microsphere's emissions at 670 nm, indicative of the presence of DiIC and 610 nm indicative of the presence of TRC were recorded using a 595 nm dichroic and an acquisition
20   time of 5 seconds for the CCD 236.
        Next, the distal end 212 of the fiber bundle is placed in a buffer and another image taken while illuminating the beams with 490 nm light. Emissions in the 530 nm fluorescein wavelengths were recorded with a 505 nm dichroic. In this
25   case, a CCD acquisition time of one second was used. This process provides a background normalizing image. The buffer was removed and the fiber allowed to dry to avoid substrate solution dilution.
        The substrate solution is then introduced and CCD images
30   acquired every 30 seconds to a minute for 30 minutes. While illuminating the microspheres with 490 nm light and collecting emissions in the 530 nm range. Fiber is then

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placed back in the buffer solution and another background
image captured. Those beads that generate a signal
indicative of fluorescein production are decoded. Depending
in the ratio of the intensity of light from the two reporter
5   dyes, DiIC:TRC, the chemical functionality of the optically
active beads may be decoded according to the following table.

0.617 - 1.08	alkaline phosphatase bead
3.188-5.725	β-galactosidase bead
0.183 - 0.243	β-glucunonidese bead

This process is then repeated for the remaining two
10 substrates.
      Figs. 8A-8C are images generated by the CCD 236 when the
bead populations are exposed to fluorescein di-phosphate.
Fig. 8A illustrates the signals from the alkaline phosphatase
microspheres when excited at 490 nm and recording emissions
15 at 530 nm, emissions at this wavelength being indicative of
fluorescein production. Fig. 8B shows the image captured by
the CCD when the microspheres are excited at 577 nm and
emissions at 670 nm are recorded. This wavelength is an
encoding wavelength indicative of the concentration of DiIC
20 on the microspheres. Finally, Fig. 8C shows the image when
the microspheres are excited with 577 nm light and emissions
in the 610 nm range are recorded being indicative of the
concentration of TRC in the microspheres.
      In a similar vein, Figs. 9A and 9B are images when the
25 microspheres are exposed to fluorescein d-β-galactosidase.
Fig. 9A shows emissions at 530 nm indicative of the
fluorescein production; and Fig. 9B shows light emitted at
the 670 nm range indicative of the presence of DiIC.

27

These micrographs, Fig. 8A-8C and 9A-9B illustrate fluorescein production around the microspheres may be detected as an optical signature change indicative of reactions involving the chemical functionality of the
5 microspheres. The micrographs also illustrate that the optical signatures may be decoded to determine the chemical functionalities on each microsphere.

2. Genosensor:

Three separate subpopulations of beads were used. In
10 subpopulation A, xrabbit antibodies (Ab) were affixed to the surface of the microspheres; in subpopulation B, xgoat antibodies were affixed to the microspheres; and in subpopulation C, xmouse antibodies were affixed to the microspheres. These three separate subpopulations were
15 identified using a DiIC:TRC encoding ratio similar to that in the previously described experiment.

For the first step of the experiment, images at the encoded wavelengths were captured using 577 nm excitation and looking for emissions at 610 and 670 nm. After this
20 decoding, the fiber was placed in a buffer and an image taken at 530 nm with 490 nm excitation. This provided a background normalizing signal at the fluorescein emission wavelength. Next, the fiber was placed in rabbit IgG antigen (Ag) which is fluorescein labeled. Images were then captured every few
25 minutes at the 530 nm emission wavelength for fluorescein. Figs. 10A and 10B are micrographs showing the image captured by the CCD prior to and subsequent to exposure to a rabbit antigen, which clearly show reaction of the selected microspheres within the population.

30 Note, if the fluorescein background from the antigen solution is too high to see the antibody-antigen signal, the fiber bundle may be placed in a buffer. This removes the background florescence leaving only the Ab-Ag signal.

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While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein
5   without departing from the spirit and scope of the invention as defined by the appended claims.

29

CLAIMS

What is claimed is:

5   1.   An analytic chemistry system, comprising a population of
           beads including separate subpopulations, each
           subpopulation carrying chemical functionality which
           changes an optical signature of the beads in the
           presence of targeted analytes, beads in each
10   subpopulation having an optical signature which is
           encoded with a description of the chemical functionality
           carried by that subpopulation.

     2.   The system described in Claim 1, wherein the beads are
15   encoded using dyes.

     3.   The system described in Claim 2, wherein the dyes are
           entrapped within the beads and the chemical
           functionality is on surfaces of the beads.
20
     4.   The system described in Claim 1, wherein the beads are
           encoded using fluorescent dyes.

     5.   The system described in Claim 1, wherein the beads are
25   encoded by controlling a ratio of at least two dyes.

     6.   The system described in Claim 1, wherein the chemical
           functionality changes the optical signature by producing
           an optically active chemical in the presence of targeted
30   analytes.

     7.   The system described in Claim 1, wherein the optical
           signature is changed by the chemical functionalities of

30

the beads by the presence or absence of a fluorescent
signal.

8.   The system described in Claim 1, wherein the chemical
5   functionalities of the beads support sites for
       hybridization.

9.   The system described in Claim 1, wherein the beads are
       affixed to a distal end of an optical fiber bundle.
10

10.   The system described in Claim 1, wherein the beads are
       located within etched wells at terminal ends of optical
       fibers of the bundle.

15 11. A chemical analysis method, comprising
                preparing separate subpopulations of beads,
           each subpopulation carrying chemical
           functionalities that change optical signatures of
           the beads in the presence of targeted analytes;
20   encoding optical signature of the beads in
           each subpopulation with a description of the
           chemical functionalities carried by that
           subpopulation;
                combining the subpopulations to produce a
25   system;
                applying the system;
                detecting changes in the optical signatures
           indicative of a presence of the targeted analytes;
           and
30   decoding optical signature of the beads to
           identify the chemical functionalities.

31

12.   The method described in Claim 11, wherein encoding the
      optical signatures with the chemical functionalities
      comprises doping the beads with fluorescent dyes.

5 13.   The method described in Claim 11, wherein encoding the
      optical signatures with chemical functionalities
      comprises attaching encoding dyes to the beads.

14.   The method described in Claim 11, wherein encoding the
10   optical signatures with the chemical functionalities
      comprises controlling a ratio of at least two dyes
      carried by each bead.

15.   The method described in Claim 11, further comprising:
15   encoding the beads with the chemical
      functionalities by entrapping dyes within or
      attaching dyes to the beads; and
         applying the chemical functionalities to the
      beads.

20

16.   The method described in Claim 11, further comprising
      enabling the chemical functionalities to produce an
      optically active species in the presence of targeted
      analytes to change the optical signature.

25

17.   The method described in Claim 11, further comprising
      changing the optical signature by the presence or
      absence of a fluorescent signal from the beads.

30 18.   The method described in Claim 11, further comprising
      enabling the chemical functionalities to hybridize.

19.   An analytic chemistry sensor, comprising:

32

        a bundle of optical fibers;

        a population of beads carrying chemical
    functionalities at a distal end of the fiber optic
    bundle, light from individual bead being coupled
5   into separate or groups of separate fibers of the
    bundle for transmission to the proximal end of the
    bundle.

    20. The sensor described in Claim 19, wherein each one of
10   the beads is located within separate wells formed at
         terminal ends of optical fibers of the bundle.

    21. The sensor described in Claim 20, wherein the wells are
         formed by anisotropic etching of the cores of the
15   optical fibers with respect to the cladding.

    22. The sensor described in Claim 19, further comprising a
         light source for exciting optically active chemicals
         bound to the chemical functionalities.
20
    23. The sensor described in Claim 19, wherein the population
         of beads includes separate subpopulations, each
         subpopulation carrying a different chemical
         functionality and an optically interrogatable code
25   descriptive of the chemical functionality.

    24. The sensor described in Claim 23, further comprising a
         light source for exciting optically active chemicals
         bound to the chemical functionalities.
30
    25. The sensor described in Claim 23, wherein code of each
         subpopulation comprises fluorescent dyes.

33

26. The sensor described in Claim 23, further comprising a
     filter and a frame capturing camera for detecting
     optical signatures indicative of a status of the
     chemical functionalities and optical signatures
5   indicative of the encoding of the beads.

27. A method for constructing and using an analytic
     chemistry sensor, comprising:
               forming wells at terminal ends of optical
10   fibers within a bundle;
             distributing beads carrying chemical
         functionalities within the wells; and
               monitoring a status of the chemical
         functionalities from a proximal end of the bundle.
15

28. The method described in Claim 27, wherein forming the
     wells comprises anisotropically etching of cores of the
     optical fibers with respect to cladding.

20 29. The method described in Claim 27, further comprising
     forming a population of beads in the wells from separate
     subpopulations, each subpopulation carrying a different
     chemical functionality and an optically interrogatable
     code descriptive of the chemical functionality.
25

30. The method described in Claim 29, further comprising
     randomly distributing the subpopulations within the
     wells.

30 31. The method described in Claim 29, further comprising
     serially adding the subpopulations to the wells.

Fig. 1

```
                                              ┌─50
        ┌──────────────────────────┐
        │   VACUUM FILTER SELECTED  │
        │       MICROSPHERES        │
        └──────────────────────────┘
                     │
                     ▼                        ┌─52
        ┌──────────────────────────┐
        │      ADD DYE SOLUTION     │
        └──────────────────────────┘
                     │
                     ▼                        ┌─54
        ┌──────────────────────────┐
        │     REMOVE EXCESS DYE     │
        └──────────────────────────┘
                     │
                     ▼                        ┌─56
        ┌──────────────────────────┐
        │     ATTACH CHEMICAL       │
        │      FUNCTIONALITY        │
        └──────────────────────────┘
```

FIG. 2

Fig. 3

Fig. 4

Fig. 5A

Fig.5B

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PREPARE BUNDLE ENDS — 270

ETCH DISTAL END — 272

STOP ETCH AND CLEAN — 274

PLACE MICROSPHERES IN WELLS — 276

FIX MICROSPHERES IN WELLS — 278
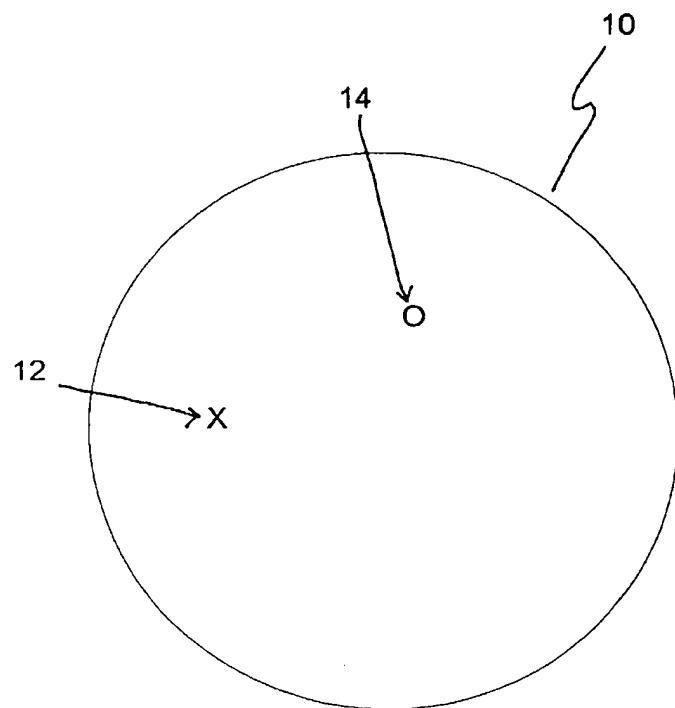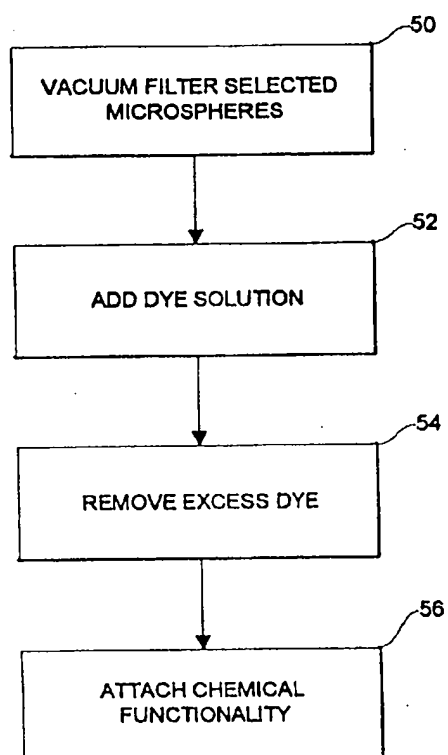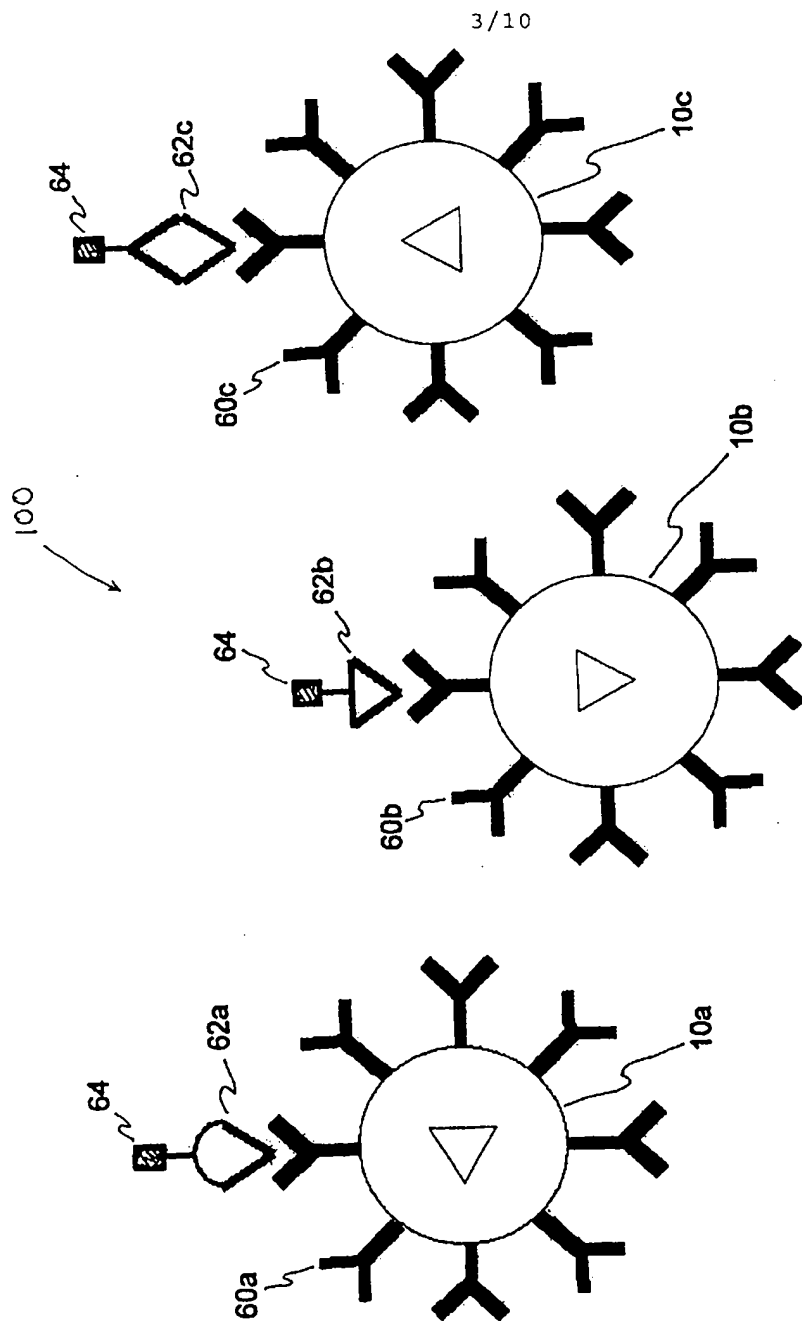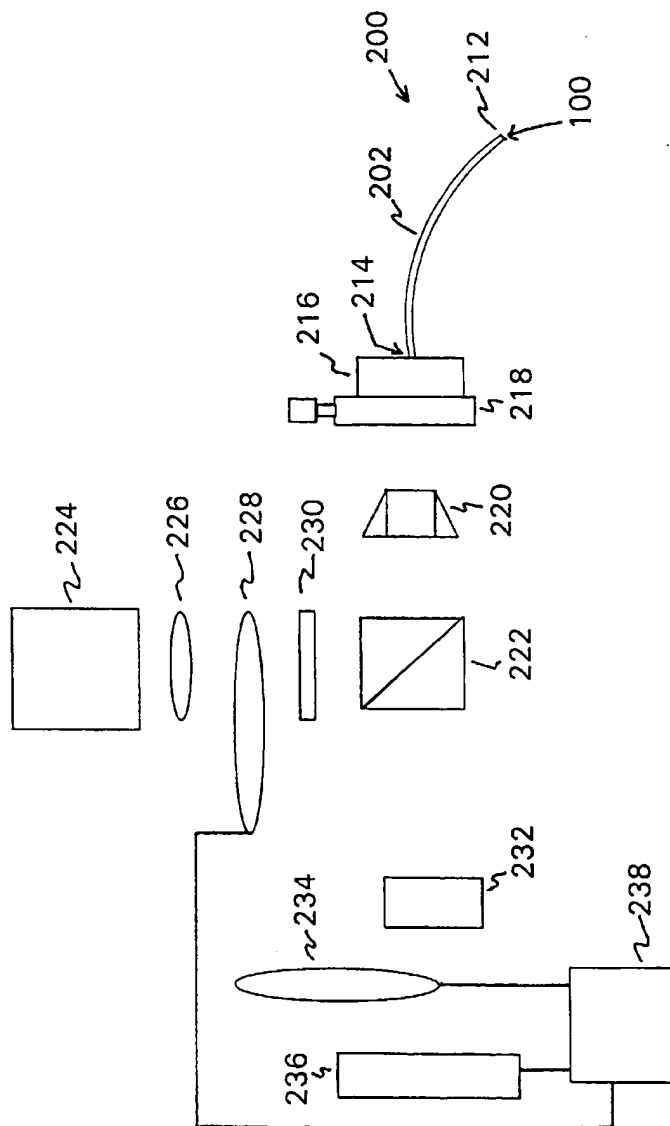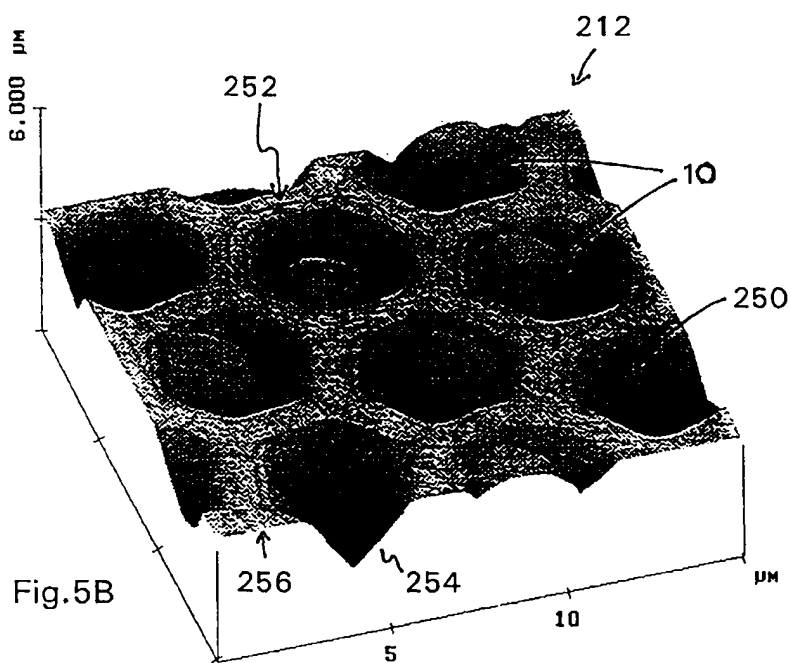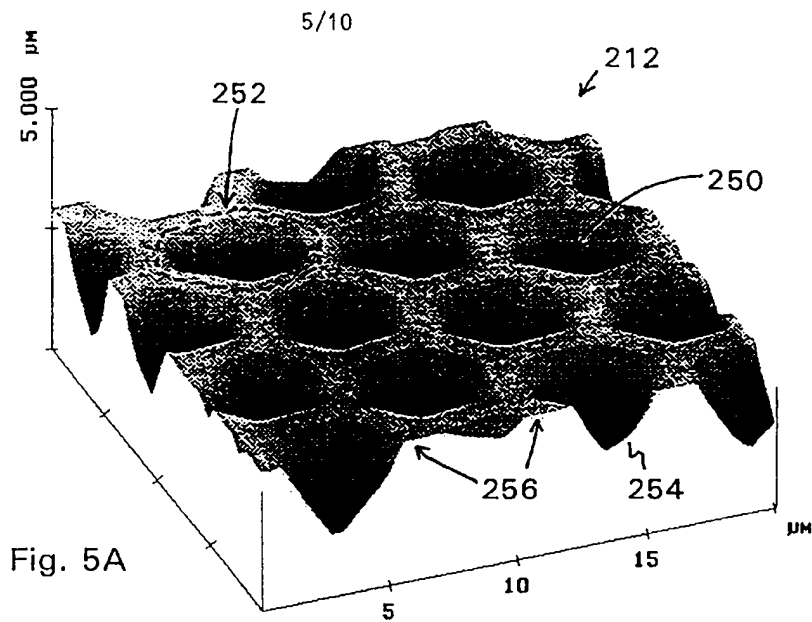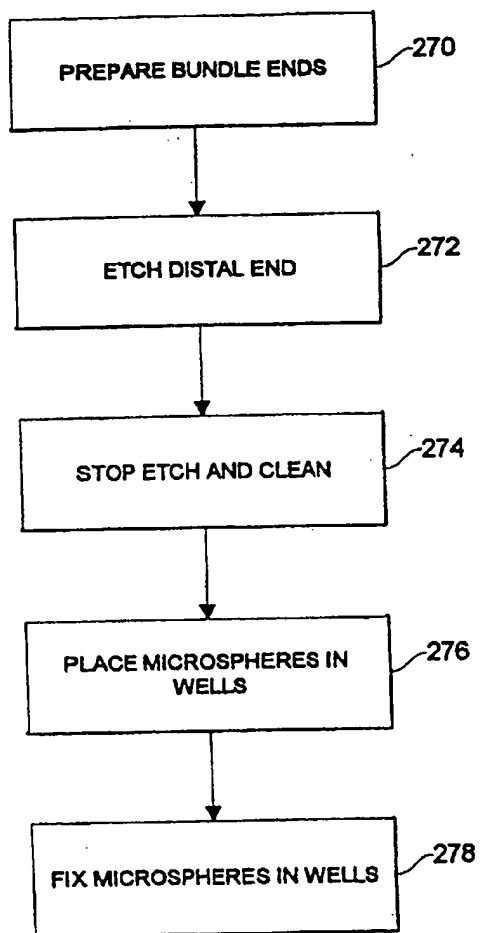
FIG. 6
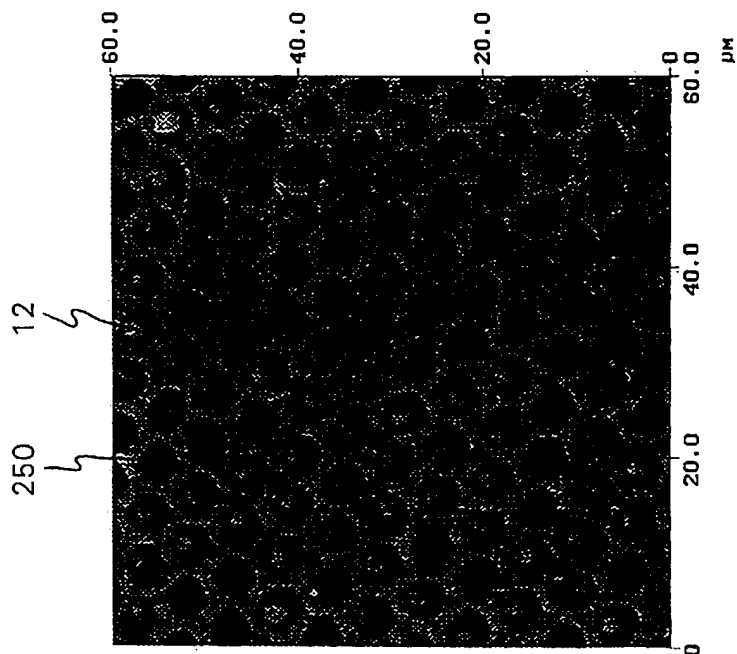
Fig. 7B
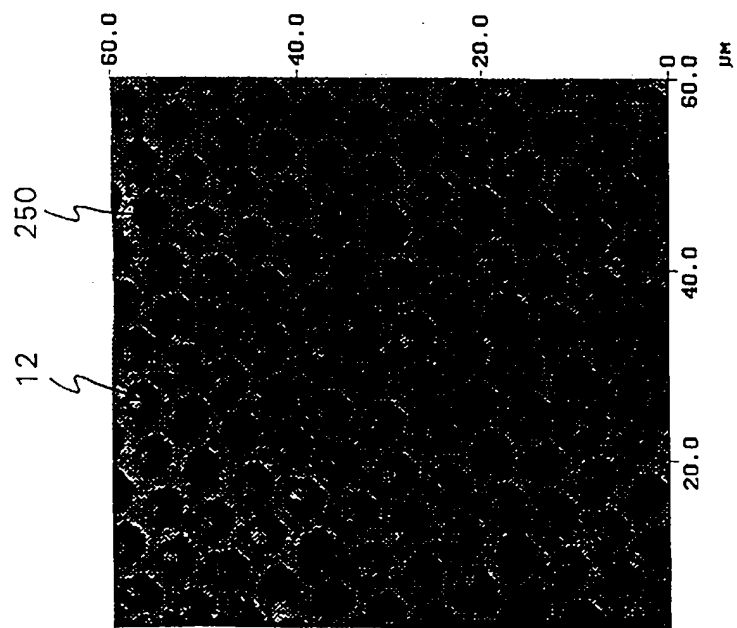
Fig. 7A
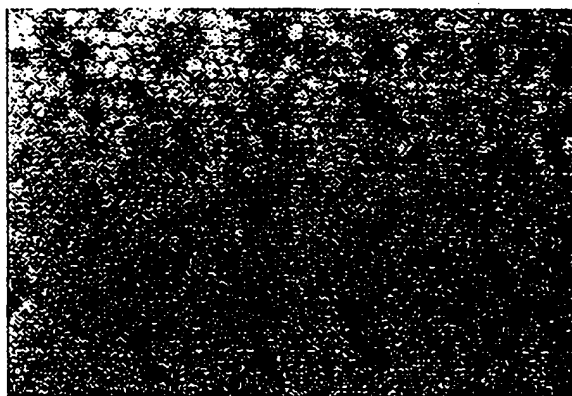
RECTIFIED SHEET (RULE 91)
ISA/EP
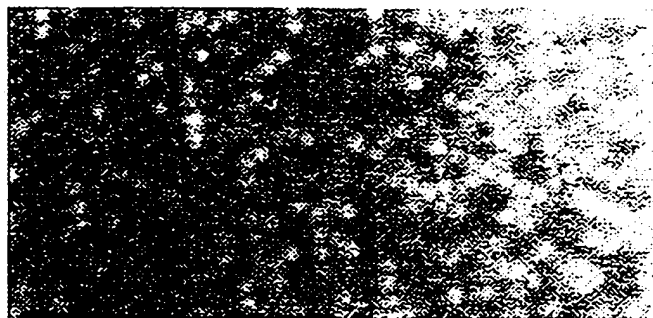
Fig. 8A
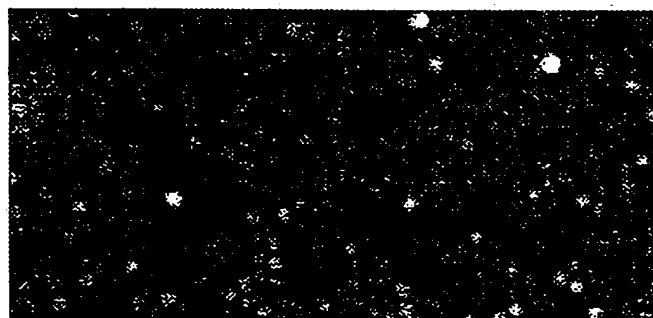
Fig. 8B
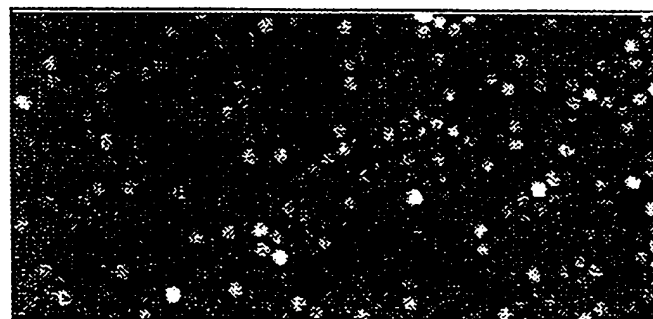
Fig. 8C

Fig. 9A
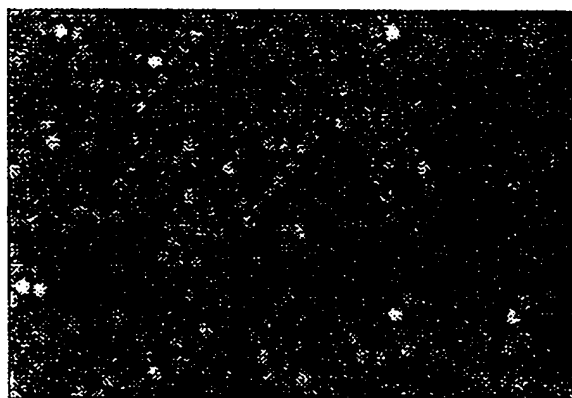
Fig. 9B

RECTIFIED SHEET (RULE 91)
ISA/EP
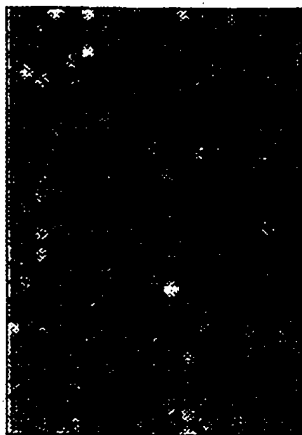
Fig. 10B
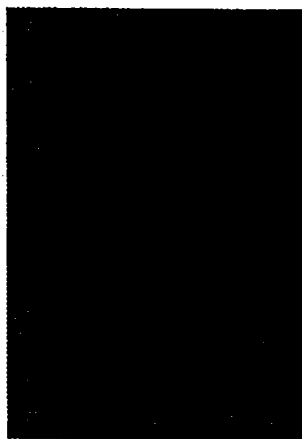
Fig. 10A

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N21/77   G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 14028 A (LUMINEX CORP) 17 April 1997 see abstract ---	1-8
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X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
13 August 1998	24/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Zinngrebe, U

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